PB86-129277

Hazard Evaluation Division, Standard Evaluation Procedure: Acute Toxicity Test for Freshwater Fish

(U.S.) Environmental Protection Agency
Washington, DC

Jun 85

U.S. Department of Commerce
National Technical Information Service
NTIS

PB86-129277

EPA-540/9-85-006
June 1985

HAZARD EVALUATION DIVISION

STANDARD EVALUATION PROCEDURE

ACUTE TOXICITY TEST FOR FRESHWATER FISH

Prepared by

Elizabeth Zucker, M.S.

Standard Evaluation Procedures Project Manager
Stephen L. Johnson
Hazard Evaluation Division
Office of Pesticide Programs

United States Environmental Protection Agency
Office of Pesticide Programs
Washington, D.C. 20460

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

50272-101

REPORT DOCUMENTATION PAGE	1. REPORT NO.	2.	3. Recipient's Accession No. PB86 129277/AS

4. Title and Subtitle
HAZARD EVALUATION DIVISION, STANDARD EVALUATION PROCEDURE
Acute Toxicity Test for Freshwater Fish

5. Report Date
June 1985

6.

7. Author(s)
Elizabeth Zucker

8. Performing Organization Rept. No.
EPA-540/9-85-006

9. Performing Organization Name and Address
U.S. Environmental Protection Agency/OPP/HED/(TS-769C)
401 M Street SW
Washington, D.C. 20460

10. Project/Task/Work Unit No.

11. Contract(C) or Grant(G) No.
(C)
(G)

12. Sponsoring Organization Name and Address
Same as #9.

13. Type of Report & Period Covered

14.

15. Supplementary Notes
Supplement to Pesticide Assessment Guidelines Subdivision E - Hazard Evaluation: Wildlife and Aquatic Organisms (EPA-540/9-82-024 and NTIS Order Number PB83-153908), October, 1982.

16. Abstract (Limit: 200 words)

The Standard Evaluation Procedure (SEP) for the Acute Toxicity Test for Freshwater Fish is a guidance document primarily intended for Agency reviewers and the regulated industry who evaluate ecological effects data specified in 40 CFR Part 158.145. The SEP is also intended to provide information to the general public indicating how the Agency evaluates these types of studies. As such, it is designed to supplement Subdivision E of the Pesticide Assessment Guidelines: Hazard Evaluation - Wildlife and Aquatic Organisms. This SEP provides an Introduction, Materials and Methods, Reporting Requirements, Reviewer Evaluation, and Appendix of appropriate methods to guide the review and scientific evaluation of pesticide effects on fish.

The SEP for the Acute Toxicity Test for Freshwater Fish is only one of a number of SEP's published by the National Technical Information Service as a supplement to Subdivision E of the Pesticide Assessment Guidelines.

17. Document Analysis a. Descriptors

b. Identifiers/Open-Ended Terms

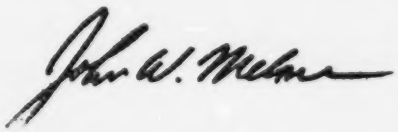
c. COSATI Field/Group

18. Availability Statement	19. Security Class (This Report)	21. No. of Pages
Unclassified and freely available.	Unclassified.	18
	20. Security Class (This Page) Unclassified.	22. Price

STANDARD EVALUATION PROCEDURE

PREAMBLE

This Standard Evaluation Procedure (SEP) is one of a set of guidance documents which explain the procedures used to evaluate environmental and human health effects data submitted to the Office of Pesticide Programs. The SEPs are designed to ensure comprehensive and consistent treatment of major scientific topics in these reviews and to provide interpretive policy guidance where appropriate. The Standard Evaluation Procedures will be used in conjunction with the appropriate Pesticide Assessment Guidelines and other Agency Guidelines. While the documents were developed to explain specifically the principles of scientific evaluation within the Office of Pesticide Programs, they may also be used by other offices in the Agency in the evaluation of studies and scientific data. The Standard Evaluation Procedures will also serve as valuable internal reference documents and will inform the public and regulated community of important considerations in the evaluation of test data for determining chemical hazards. I believe the SEPs will improve both the quality of science within EPA and, in conjunction with the Pesticide Assessment Guidelines, will lead to more effective use of both public and private resources.



John W. Melone, Director
Hazard Evaluation Division

TABLE OF CONTENTS

	Page
I. INTRODUCTION	
A. When Required	1
B. Purpose	1
C. Test Material	1
1. Technical Grade	1
2. End-Use Product	2
II. MATERIALS AND METHODS: TESTING STANDARDS/DATA ACCEPTABILITY	
A. Recommended Protocols	2
B. Test Organisms	3
1. Acceptable Species	3
2. Size/Age/Physical Condition	3
3. Source/Acclimation	4
C. Test System	4
1. Source of Dilution Water	4
2. Temperature	5
3. Test Vessels	5
4. Photoperiod	5
5. Loading	5
6. Solvents	6
D. Test Design	6
1. Test Levels	6
2. Number of Test Animals	6
3. Controls	6
4. Beginning the Test	7
5. Measuring Temperature/DO/pH	7
6. Chemical Analysis	7
III. REPORTING REQUIREMENTS	
A. Test Material	8
B. Dilution Water/Test Vessels	8
C. Test Organisms	8
D. Range Finding Tests	8
E. Definitive Tests	9
F. Calculated LC50	9
G. Temperature/DO/pH	9
H. Chemical Analysis	9
I. Testing Protocols	9
IV. REVIEWER'S EVALUATION	
A. Review of Test Conditions	9
B. Verification of Statistical Analyses	9

TABLE OF CONTENTS (Continued)

	Page
C. Conclusions	10
1. Categorization of Results	10
2. Rationale	11
3. Repairability	11
D. Descriptive Classification	11
E. References	12

ACUTE TOXICITY TEST FOR FRESHWATER FISH

I. INTRODUCTION

A. When Required

Acute toxicity testing on freshwater fish is required to support registration of manufacturing-use pesticide products and end-use pesticide products intended for outdoor application.

B. Purpose

Acute toxicity studies on freshwater fish determine the lethal concentration (LC_{50}) of a chemical which will kill fifty percent of the test population in 48 to 96 hours. These acute tests have attained broad acceptance among environmental toxicologists as relatively rapid, uncomplicated, inexpensive, and statistically reliable methods for assessing immediate, short-term, adverse effects of chemicals on fish.

The Ecological Effects Branch regularly requires that results of two freshwater fish acute toxicity tests be submitted to support the registration of a pesticide. One study should be performed on a coldwater fish species and one study on a warmwater fish species. The data from these tests are used:

- To establish acute toxicity levels of the active ingredient to nontarget freshwater fish;
- To assess potential impact to fish by comparing toxicity information with measured or estimated pesticide residues in the freshwater environment;
- To provide support for precautionary label statements that will minimize adverse effects to fish when the pesticide is used according to directions; and
- To indicate the need for further laboratory testing and/or field studies.

C. Test Material

1. Technical Grade

Tests must be conducted with the technical grade of the active ingredient. If more than one active ingredient constitutes a technical product, then the technical grade of each active ingredient must be tested separately.

2. End-Use Product

In addition to technical product testing, the applicant may be required to test the end-use product as well if:

- The end-use product will be introduced directly into an aquatic environment when used as directed;
- The freshwater fish LC_{50} of the technical grade of the active ingredient is equal to or less than the expected environmental concentration in the freshwater environment when the end-use product is used as directed;
- An ingredient of the formulated end-use product is expected to enhance the toxicity of the end-use product beyond that expected from the active ingredient(s) alone; or
- The technical product is insoluble in water but the formulated product is soluble in water. In this situation, the test design should include a control where organisms are exposed to just the inert ingredients and carriers.

II. MATERIALS AND METHODS: TESTING STANDARDS/DATA ACCEPTABILITY

A. Recommended Protocols

Because the acute test is an established technique for assessing toxicity of a chemical to freshwater fish species, much of the methodology for performing these studies, as well as the procedures for statistical analyses of results, have been carefully outlined and documented in the published literature. Notably, the information to be discussed in this Standard Evaluation Procedure (SEP) is presented in greater detail in the following references:

Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/375-009. 61 pp.

American Society for Testing Materials. 1980. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. E 729-80. Published by ASTM Committee on Standards, 1916 Race Street, Philadelphia, PA, 19103.

Peltier, William. 1978. Methods for Measuring the Acute Toxicity of Effluents to Aquatic Organisms. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 600/4-78-012. 52 pp.

U.S. Environmental Protection Agency. Pesticide Assessment Guidelines Subdivision E. Hazard Evaluation: Wildlife and Aquatic Organisms. EPA-540/9-82-024.

These referenced protocols are presented as flexible guidance to help researchers design scientific protocol and to help the reviewer validate studies. It is important to recognize that freshwater fish tests are validated as to whether they provide scientifically sound information on the acute toxicity of the test material to fish and whether the results of the study will fulfill guideline requirements. This is more important than whether a study completely conforms to referenced protocols. It is sometimes necessary to alter the procedures presented in published protocols to meet the characteristics of the chemical or test organisms used.

The static test is the standard technique for obtaining LC_{50} values for fish, however flow-through testing may be needed when toxicants are highly volatile or otherwise unstable in the aqueous environment, or when a chemical has a high biochemical oxygen demand. The information as it is presented below will focus on static testing protocols. Specific references to acceptable flow-through methods are indicated when necessary.

B. Test Organisms

1. Acceptable Species

The preferred test species for the coldwater fish toxicity test is the rainbow trout (Salmo gairdneri). The bluegill sunfish (Lepomis macrochirus) is the recommended species for warmwater fish toxicity testing. Other acceptable species include:

Coldwater species
- Brook trout (Salvelinus fontinalis)
- Coho salmon (Oncorhynchus kisutch)

Warmwater species
- Channel catfish (Ictalurus punctatus)
- Fathead minnow (Pimephales promelus)

2. Size/Age/Physical Condition

Individual fish should weigh between 0.5 and 5 grams and should be of the same year class. The standard length of the longest fish should not be more than twice the length of the shortest fish. Very young (not actively feeding), sexually mature, spawning, or recently spent fish should not be utilized.

3. Source/Acclimation

All organisms must be from the same source. This may include laboratory or commercial stocks. Animals captured in the wild are acceptable provided they meet the requirements pertaining to physical condition and age/size criteria mentioned above. Organisms captured via chemical treatment must not be used. When test animals are brought into the laboratory, they should be quarantined for at least seven days. It is recommended that fish be acclimated to study conditions for at least two weeks prior to testing.

Test organisms must be observed prior to testing for signs of disease, stress, physical damage, and mortality. Injured, dead and abnormal individuals must be discarded. If the organisms show signs of disease or stress, remedial action must be taken during the acclimation period and not during the test phase.

Organisms that have been treated for disease are acceptable for testing provided they do not exhibit signs of disease during the 48-hours pretest. Organisms must not be used if they appear to be diseased or stressed or if more than 3% die during the 48 hours immediately prior to testing.

If possible, feeding of the fish should be limited to the time just prior to testing. This reduces the amount of organic material generated in the test vessels. Organic matter may alter the test results by adsorbing the chemical or increasing the oxygen demand of the test solution.

C. Test System

1. Source of Dilution Water

Whenever possible, soft, reconstituted water should be used for freshwater studies. Reconstituted water should be aged one or two weeks and intensely aerated prior to use. Detailed descriptions of acceptable procedures for preparing diluent are found in the protocols by the American Society for Testing Materials (1980) or the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975).

Use of a natural dilution water with a hardness of 40 to 48 mg/L as $CaCO_3$ can be used in lieu of reconstituted water. If possible, natural dilution water should be obtained from an uncontaminated well, spring, or surface water source. Dechlorinated water should not be used because removal of chlorine is rarely complete and residual chlorine can be quite toxic to aquatic organisms.

The dilution water must be able to support the test animals without observable signs of stress such as discoloration or

unusual behavior.

2. Temperature

The recommended temperature for testing coldwater fish species is 12°C. Warmwater fish tests should be performed at 17°C or 22°C.

Testing facilities should have a constant temperature area or a recirculating water bath for the test vessels.

3. Test Vessels

Test containers should be constructed from welded stainless steel or glass. Vessels with dimensions of about 300 x 600 x 300 mm depth are acceptable for both static and flow-through tests. Frequently, static tests are performed in 18.9 L (5 gallon) wide-mouth glass jars containing 15 L solution.

If test vessels are constructed from materials other than glass or stainless steel, solutions must be analyzed to determine exact toxicant concentrations. Past studies have shown that some test vessel materials (e.g., polyethylene) can adsorb residues of the pesticide being tested.

The metering system chosen for flow-through studies must reproducibly supply appropriate toxicant concentrations at a consistent flow rate. Metering systems should be calibrated before and after each study and checked twice daily during the test period. Flow rates should be five to ten volume additions per 24-hours. Systems should be constructed so that the organisms are not stressed by turbulence.

4. Photoperiod

A 16-hour light and an 8-hour dark photoperiod with a 15- to 30-minute transition period between light and dark is recommended.

5. Loading

The size of the test container should be such that the loading factor (test organism mass per volume of test solution) is no greater than 0.8 g/L in static tests performed at or below 17°C. At higher temperatures, a biological loading of 0.5 g/L of test solution is acceptable. For flow-through tests, the loading should be no greater than 1 g/L of solution passing through the chamber in 24 hours, and must not exceed 10 g/L of test solution at any time at or below 17°C or 5 g/L at higher temperatures.

6. Solvents

Whenever possible, the toxicant should be introduced into the test solution without the use of solvents other than water. If alternative solvents are necessary, they should be used sparingly, not to exceed 0.5 ml/L in any static test solution and 0.1 ml/L under flow-through conditions. The following solvents are preferred:

dimethyl formamide
triethylene glycol
methanol
acetone
ethanol

D. Test Design

1. Test Levels

Initially, range finding tests may be necessary to define concentrations of the toxicant needed for definitive studies. If results from a range finding study indicate a low toxicity for the chemical, a definitive test need not be performed. However, it must be determined that the chemical will have an LC_{50} greater than 100 mg/L, by exposing at least 30 individuals to a concentration of 100 mg/L or greater.

Definitive acute toxicity tests normally are designed to include one or more control groups and a geometric series of at least five toxicant concentrations to be tested. Each designated treatment group should be exposed to a concentration of toxicant that is at least 60% of the next highest concentration.

2. Number of Test Animals

In definitive tests, at least ten test organisms should be exposed to each treatment level. Treatment groups can be divided into two or more containers. All organisms must be randomly assigned to test vessels.

3. Controls

Each test requires a concurrent control using the same dilution water and the same number of organisms per test level. If a solvent other than water is used, a solvent control should be established. The highest concentration of the solvent that was added to any of the study chambers should be tested as the control.

A study is not acceptable if more than 10% of the control organisms die during a static test or 5% during a flow-through study.

4. Beginning the Test

Static acute tests are initiated either by adding the test material to the test chambers after the fish are added or by adding the fish to the test chambers within 30 minutes after the test material is added to the dilution water.

5. Measuring Temperature/DO/pH

Temperature should be measured continuously (hourly) in at least one test vessel during the entire study period. If temperature is controlled by a water bath, measurements can be recorded every six hours. Temperature should not vary more than one degree Centigrade (C) during the entire study period.

The dissolved oxygen (DO) concentration must be measured at the beginning of the test and every 48 hours thereafter to the end of the test. Measurements should be taken from the control and the high, medium, and low concentrations as long as animals are present at those levels. The DO level during the first 48 hours should be between 60% and 100% of saturation and between 40% and 100% saturation after 48 hours. In the flow-through test, the DO concentrations in each chamber should be between 60% and 100% saturation at all times during the study.

The pH should be measured at the beginning and end of the test in the control and the high, medium, and low toxicant concentrations.

6. Chemical Analysis

It is preferred that test solutions be chemically analyzed to determine exact concentrations of pesticides. It is particularly important that residues are measured if:

- The test solutions were aerated (aeration may cause volatilization of the pesticide);
- The test material was volatile, insoluble, or precipitated out of solution;
- The test containers were not made of stainless steel or glass;
- The test chemical is known to adsorb to the test container's structural material; or
- A flow-through system is used (measurement verifies accuracy of metering system).

III. REPORTING REQUIREMENTS

The test report submitted to the Agency must fully describe the materials and methodology used to perform the study. The reviewer must be able to establish from the report that the study was performed under conditions that render the results acceptable for use in a risk assessment and/or for fulfilling the guidelines requirement. The following information is particularly important for a complete evaluation.

A. Test Material

If the study is to be performed with the technical grade product, the test material should be clearly identified as to source, batch, and exact purity. Simply identifying the material as technical may not be acceptable because the percent active ingredient of some newer products may increase with time as the manufacturing process is improved to produce greater yield.

For studies involving the end-use product, the exact percent of the active ingredient and the type of formulation (e.g., granular, wettable powder) of the test material should be described. It should be clearly stated in the test report whether results are expressed in terms of active ingredient or as total formulated product.

B. Dilution Water/Test Vessels

Test reports submitted to the Agency should include a complete description of dilution waters used in the toxicity studies. Descriptions should include identification of the source, the chemical characteristics of the water, and information on any pretreatments.

Test vessels should be described as to construction material and size, as well as depth and volume of solution.

C. Test Organisms

Test reports should provide complete descriptions of source, holding, and acclimation conditions including information on feeding schedules and disease treatment procedures.

The age, average weight, and length of test fish should be reported. Species should be identified by scientific name.

D. Range Finding Tests

Test reports should provide information describing range finding study procedures and results. The information should include sample sizes, concentrations tested, and mortality data.

E. Definitive Tests

Procedures used to prepare toxicant stock solutions and test aliquots should be thoroughly described.

The criteria for determining effects must be defined. The raw data or percentage of deaths at each level as well as the number of fish tested per level must be reported for each 24-hour period of the study. Toxic symptoms (physical and behavioral) should be described throughout the test period.

F. Calculated LC50

The statistically calculated LC_{50} with 95% confidence limits and method of calculation must be presented. The slope of the dose-response line should be calculated and reported.

In lieu of a calculated LC_{50}, the study may show that the LC_{50} is greater than 100 ppm.

G. Temperature/DO/pH

Dissolved oxygen and pH measurements should be reported along with the range and average temperature.

H. Chemical Analyses

If chemical analyses are conducted, the test report should provide information on the methods (or references to established methods) utilized and results of analyses. Residues found at the beginning and end of the study should be reported.

I. Testing Protocols

The test report should include references to any protocols followed during the test.

IV. REVIEWER'S EVALUATION

A. Review of Test Conditions

The reviewer should note any important information missing from the submitted report. Also noted are conditions of the study that are inconsistent with recommended methodologies as discussed in this SEP or in designated references.

B. Verification of Statistical Analyses

An integral part of the data evaluation process is the verification of statistical analyses. The reviewer should ensure that the LC_{50} has been properly derived by recalcuating data through currently available statistical programs.

An acceptable acute toxicity test should provide additional important information other than the LC_{50} (EC_{50}). Results from a valid study should provide a zero mortality level and a slope of the dose-mortality response line. These data can give further insight into the toxicological characteristics of the chemical such as whether the response is gradual over a wide concentration range or relatively rapid over a narrow range.

If the recalculated results differ substantially from the submitted results, the reviewer should note this and attempt to explain the discrepancies.

A test can be considered unacceptable if more than 10% of the control organisms die during the study period. An inadequate number of test organisms per test level can also produce unreliable results.

C. Conclusions

1. Categorization of Results

The significance of inconsistencies in the test procedures must be determined by the reviewer so that the results of the study can be categorized as to their usefulness in a risk assessment. Categories are described as:

° Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard methodologies may be apparent; however, the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of Part 158 of the regulations and are acceptable for use in a risk assessment.

° Supplemental: Studies in this category are scientifically sound; however, they were performed under conditions that deviated substantially from recommended guideline protocols. Results do not meet regulatory requirements; however, the information may be useful in a risk assessment.

Some of the conditions that may place a study in a supplemental category include:

- Unacceptable test species;
- Inappropriate test material;
- Dosage levels tested were less than 100 ppm but not high enough to produce an effect on the organisms or a precise LC_{50} (EC_{50}); or
- Deviations from recommended test solution characteristics (variations in DO, temperature, hardness, and pH can affect toxicological response).

° Invalid: These studies provide no useful information. They are not scientifically sound, or they were performed under conditions that deviated so significantly from recommended protocols that the results will not be useful in a risk assessment.

Examples of studies placed in this category commonly include those where the test system was aerated, test vessels were constructed from materials other than glass, or there were problems of solubility or volatility of the test material. Unless acceptable chemical analyses of actual toxicant concentrations were performed in studies such as these, the reviewer cannot be sure that organisms were actually exposed to nominally designated residues. Also, a study where the test material was not properly identified can be invalidated.

2. Rationale

To support a supplemental or invalid category, the reviewer must list and explain all test conditions that deviated from standard protocols.

3. Repairability

If any or all of the deviations can be re-examined and found acceptable (i.e., the study category can be upgraded), the reviewer also discusses this. Usually to upgrade a study additional information must be acquired.

D. Descriptive Classification

Valid aquatic LC_{50} (EC_{50}) toxicity values can be categorically compared to LC_{50} (EC_{50}) values determined for other chemicals and/or species by the following descriptive classification:

LC_{50} (EC_{50}) (ppm)	Category Description
< 0.1	very highly toxic
$0.1 - 1$	highly toxic
$> 1 \leq 10$	moderately toxic
$> 10 \leq 100$	slightly toxic
> 100	practically non-toxic

These descriptive categories are for inter-chemical comparisons only and do not reflect actual environmental hazard to freshwater organisms.

E. References

The reviewer should reference any information used in the validation procedure. This should include protocol documents, statistical methods, or information taken from files of other division branches.